

# High altitude morphotype of the widespread *Lobrathium multipunctum* (Gravenhorst, 1802) (Coleoptera, Staphylinidae, Paederinae) revealed by DNA-barcoding

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## Abstract

High altitude ecosystems are often home to endemic species that have evolved in isolation from their low elevation counterparts. In many cases, especially in insects, such mountain endemics are often apterous (due to their reduced ability to fly and disperse). In most cases, so far, these mountain endemics are genetically differentiated from lowland sister species or populations. During an excursion in Central Spain, we encountered two such strikingly different morphotypes of the rove beetle *Lobrathium multipunctum* (Gravenhorst, 1802) (Coleoptera, Staphylinidae, Paederinae). The morphotype from high elevation was smaller and paler than those at low elevation, which were slightly larger, darker and regularly coloured. The high altitude morphotype was earlier considered a separate species (*Lobrathium hispanicum* Doderö, 1916) from its widespread lower-land counterpart (*Lobrathium multipunctum* (Gravenhorst, 1802)) before their relatively recent synonymy. Using the cytochrome c oxidase subunit I (COI) barcode region, we tested whether these distinct morphotypes are distinct species. We found that their synonymy is supported, based on multiple species delimitation methods. We suggest that this phenomenon may be more widespread amongst insects and other organismal groups. We note that the presence of high altitude morphotypes which are phylogenetically nested within, or genetically identical to, widespread lowland species (regular morphotype) is rarely reported in the literature on beetles (and other insects). These findings thus highlight the need for caution when describing mountain endemics and further highlight DNA barcoding as a helpful tool for their study.

## Key Words

beetles, barcoding, mountains, endemics, COI, species delimitation, Central Spain

## Introduction

Mountains have been highlighted again and again as cradles for speciation globally. As a result, they often harbour a large number of endemic taxa (Perrigo et al. 2019). Such a pattern is observed in rove beetles (Coleoptera, Staphylinidae) where many species are isolated at higher elevations, often with restricted distributions. Several different species can thus occur on different peaks across relatively small areas. Compared to their low altitude relatives, these are generally of small size, with reduced eyes, flightless

or apterous and with lighter pigmentation (Bordoni 2020; Chatzimanolis and Brunke 2021; Reyes-Hernández et al. 2022). Similar traits can be found for species living in caves or hypogean environments (Solodovnikov and Hansen 2016; Hu et al. 2020) and those endemic to isolated islands (Jenkins Shaw and Solodovnikov 2016; Jensen et al. 2020). Like species restricted to high elevations, these hypogean or island taxa are often restricted in area of distribution, either because of habitat or space constraints.

The genus *Lobrathium* Mulsant & Rey, 1878 belongs to the subfamily Paederinae and contains 201 species

(Newton 2022) on the global scale. *Lobrathium* is known to have local endemics (Hernando 2012; Assing 2019; Anlaş 2020), but also several very widespread species (Assing 2007). As a result of this and due to its diversity in the Western Palearctic Region, the genus has received rather extensive taxonomic attention.

During fieldwork in the Extremadura Region of Spain in 2019, we collected 18 specimens of *Lobrathium* from different localities at high and low altitudes (Fig. 1). During our initial sorting to morphospecies, we considered the specimens to represent two distinct species, based on their external morphology and the distinct habitats and altitudes at which they were collected. It soon became apparent that, based on the most recent taxonomic treatment (Assing 2007), these were, in fact, considered a single species. Assing (2007) synonymised *Lathrobium* (*Lobrathium*) *hispanicum* Doderö, 1916 with *Lobrathium multipunctum* (Gravenhorst, 1802) and designated a lectotype for the former. *Lobrathium multipunctum* is a widespread and variable species, occurring in practically all of Europe and in North-West Africa (Smetana 2004; Assing 2007).

To test whether these two morphologically distinct groups of specimens represented a single or multiple species, we extracted DNA and sequenced the cytochrome oxidase I (COI) barcode region. We analysed the resulting data alongside publicly-available sequences from NCBI GenBank and BOLDSystems by using multiple phylogenetic and species delimitation methods.

are deposited at the Natural History Museum of Denmark (NHMD - Alexey Solodovnikov). All specimens used for DNA barcoding are appended with an additional blue label ‘DNA Voucher LOB<number> NHMD’ pinned with the carded voucher specimen and locality labels (Table 1).

Material of this study is deposited in the following collections:

- cAss** Personal collection of V. Assing, property of Naturhistorisches Museum Wien, Vienna, Austria (H. Schillhammer);
- cSch** Personal collection of M. Schülke, property of Museum für Naturkunde, Berlin, Germany (B. Jaeger);
- MNHN** Muséum National d’Histoire naturelle, Paris, France (N. Berti, A. Taghavian);
- MSNG** Museo Civico di Storia Naturale “G. Doria”, Genova, Italy (R. Poggi);
- MHNG** Muséum d’histoire naturelle, Genève, Switzerland (G. Cuccodoro);
- NHMD** Zoological Museum, Natural History Museum of Denmark, University of Copenhagen, Denmark (A. Solodovnikov);
- ZMHB** Museum für Naturkunde, Berlin, Germany (B. Jaeger).

Novel DNA barcoded specimens for this study

**Table 1.** Specimens of *Lobrathium multipunctum* (Gravenhorst, 1802) used in this study.

Museum ID BOLD ID	Label data	Extraction material
LOB1 LOBRA001-23	SPAIN: Guadalefra River, 4 km E of Campanario 38.8761°N, 5.5660°W, H 280 m, 9.V.2019, river bank /w reeds and grasses, general collecting, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Terminalia
LOB2 LOBRA002-23	SPAIN: Guadalefra River, 4 km E of Campanario 38.8761°N, 5.5660°W, H 280 m, 9.V.2019, river bank /w reeds and grasses, general collecting, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Terminalia
LOB3 LOBRA003-23	SPAIN: Isla de Zújar, Embalse de la Serena, 38.9107°N, 5.4259°W, h 340 m, 9.V.2019, Eucalyptus forest on river banks, sifting forest litter and flood debris, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Terminalia
LOB4 LOBRA004-23	SPAIN: Isla de Zújar, Embalse de la Serena, 38.9107°N, 5.4259°W, h 340 m, 9.V.2019, Eucalyptus forest on river banks, sifting forest litter and flood debris, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Terminalia
LOB5 LOBRA005-23	SPAIN: Sierra de Gredos, Laguna del Duque 40.2953°N, 5.7010°W, h 1900 m, 11.V.2019, subalpine meadow and shrubs, general collecting under rocks and in moss, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Hind leg
LOB6 LOBRA006-23	SPAIN: Sierra de Gredos, Laguna del Duque 40.2953°N, 5.7010°W, h 1900 m, 11.V.2019, subalpine meadow and shrubs, general collecting under rocks and in moss, leg. A.K.Hansen, J.J.Shaw, J.Kypke NHMD	Hind leg

Material and methods

Study area, specimens and depository

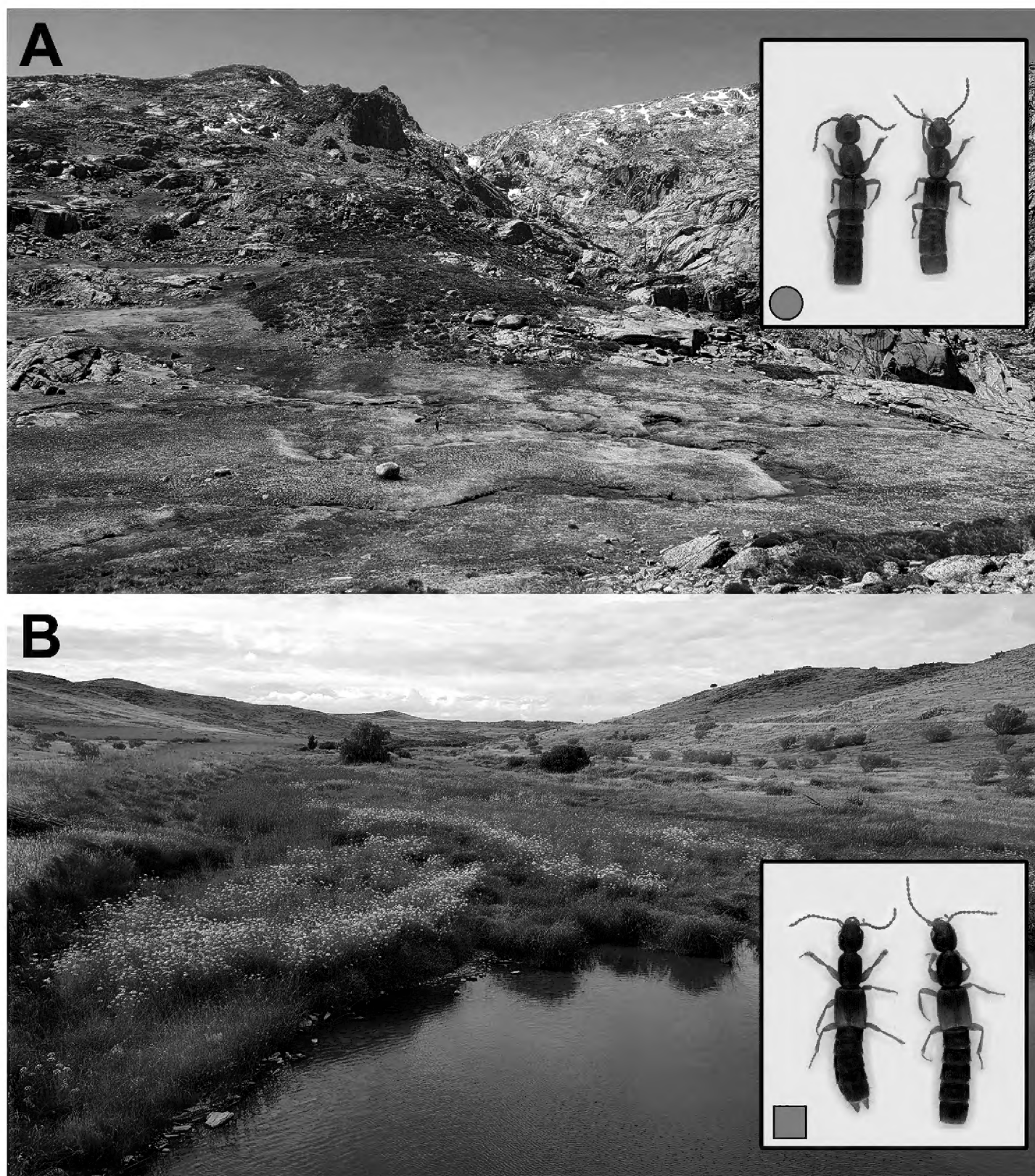
Our study area is in Central Spain in and around the Sierra de Gredos and the Tagus Basin, a granite mountain range (Sierra de Gredos) and a lush river valley (Tagus Basin). All specimens that were barcoded, examined or referenced in Assing’s (2007) revision were mapped in Figure 2. The background topographical map is based on EuroDEM elevation model. Specimens collected during the 2019 fieldtrip

Additional material

Regular morphotype of *Lobrathium multipunctum* (Gravenhorst, 1802)

SPAIN: Isla de Zújar, Embalse de la Serena, 38.9107°N, 5.4259°W, h 340 m, 9.V.2019, Eucalyptus forest on river banks, sifting forest litter and flood debris leg. A.K.Hansen, J.J.Shaw, J.Kypke (6 NHMD); Zujar River, 8.5 km NNE of Campanario 38.9301°N, 5.5672°W, H 270 m, 9.V.2019, river bank /w shrubs and trees, sifted flood debris, leg. A.K.Hansen, J.J.Shaw, J.Kypke (1





**Figure 1.** Habitat of *Lobrathium multipunctum* (Gravenhorst, 1802) (Coleoptera, Staphylinidae). **A.** High altitude morphotype (orange circle) in the Sierra de Gredos at 1900 m; **B.** Regular morphotype (purple square) at the Guadalefra River at 280 m.

NHMD); Arroyo de Navalpulo, 5 km S of Bohonal de Ibor, 39.7387°N, 5.4755°W, h 450 m, 9.V.2019, open oak forest /w grasses and creek bed, general collecting, leg. A.K.Hansen, J.J.Shaw, J.Kypke (3 NHMD); Guadalefra River, 4 km E of Campanario 38.8761°N, 5.5660°W, H 280 m, 9.V.2019, river bank /w reeds and grasses, general collecting, leg. A.K.Hansen, J.J.Shaw, J.Kypke (6 NHMD); Miajadas, [39.15°N, 5.91°W] 17.IX.1969, leg. Senglet (3 cSch); Arroyo de Jumadiel, S Brozas, [39.54°N, 6.96°W] 325 m, 20.VI.1991, leg. Wrase (4 cSch); Charca del Carrizo, S Brozas, [39.49°N, 6.75°W] 350 m, 20.VI.1991, leg. Wrase (2 cSch, 1 cAss). Ponferrada, Molaniseca [42.54°N, 6.52°W], 1.VI.1995, leg. Starke (1 cAss); Ponferrada, leg. Paganetti (1 ZMHB); Sierra de Neila, Campino, [42.04°N, 3.06°W] 1500–1900 m, 25.V.1994, leg. Schülke & Grünberg (1 cSch, 2 cAss).

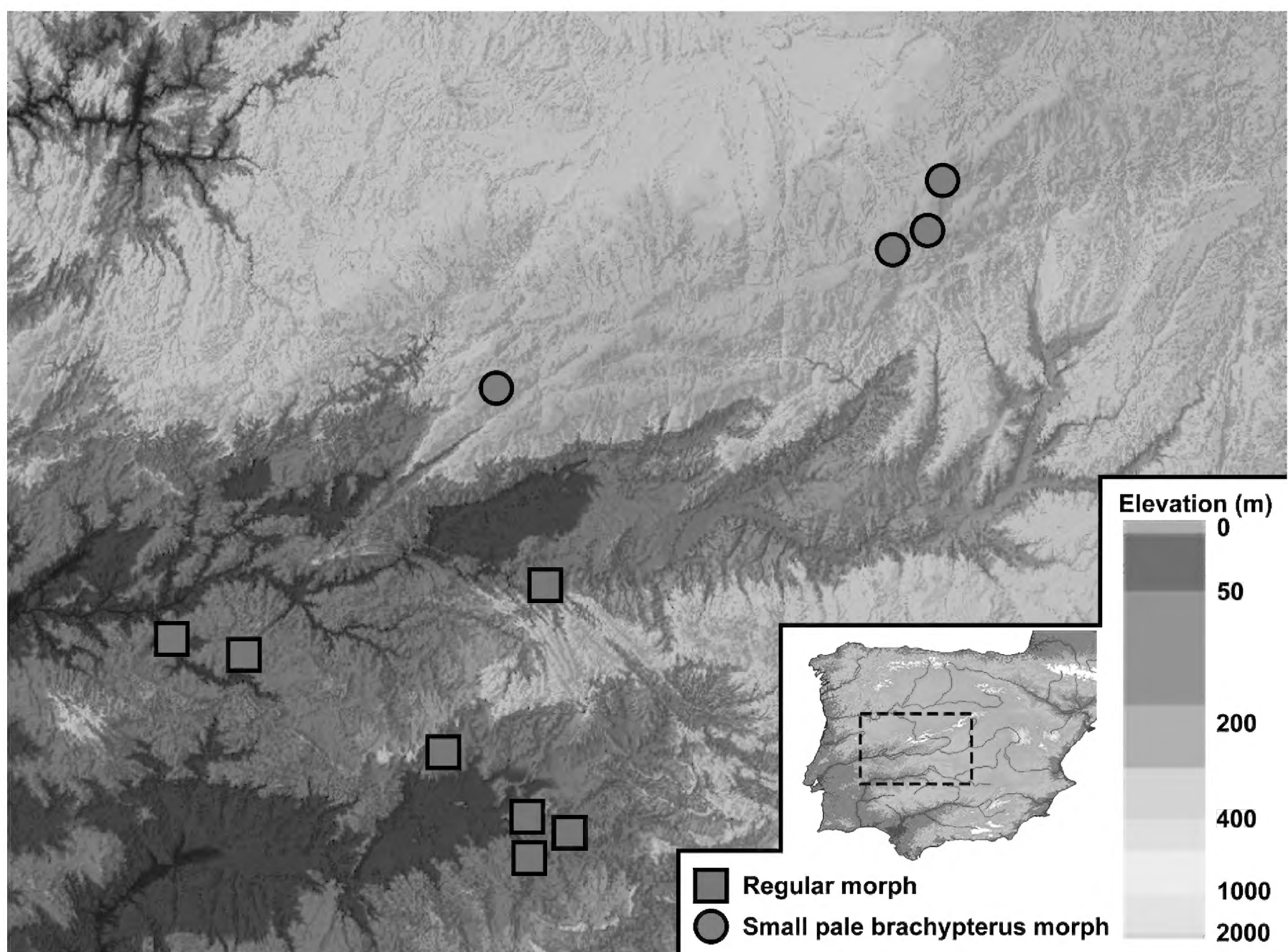
High altitude morphotype of *Lobrathium multipunctum* (Gravenhorst, 1802)

SPAIN: Sierra de Gredos, Laguna del Duque 40.2953°N, 5.7010°W, h 1900 m, 11.V.2019, subalpine meadow and

shrubs, general collecting under rocks and in moss, leg. A.K.Hansen, J.J.Shaw, J.Kypke (2 NHMD); Cercedilla [40.74°N, 4.06°W], leg. Bolivar (3 MNCN); Sierra de Guadarrama, Cabeza Lijar, [40.69°N, 4.16°W] leg. Bolivar (1 MNCN); La Granja, [40.89°N, 3.98°W], VI.1902, leg. Schramm (type of *L. hispanicum*; 3 MSNG). FRANCE: Lac Gaube [42.83°N, 0.13°W], H. P., 8.X.25 (type *L. endogeum*) (1 MNHN); [Mont] Lozère in the Massif Central [44.43°N, 3.74°E] (type *L. gallienii*).

### DNA extraction and PCR

DNA extractions were done using the EZNA DNA Tissue kit (Omega Bio-tek, Norcross, GA, USA) following the product protocol for tissue with a prolonged lysis time (16 hours). For extraction, the abdominal apex (terminalia) was detached from the specimen and used for the extraction (including segment VIII, the genital segment and, if male, the aedeagus), while for others, a hind leg was used to preserve the terminalia as intact as possible (Table 1). After extraction, the physical vouchers were



**Figure 2.** Map of known localities of *Lobrathium multipunctum* (Gravenhorst, 1802) in Central Spain of the regular morphotype (purple square) and the high altitude morphotype (orange circle).

pinned on a card mount with genital segments and aedeagus fixed on a plastic slide in euparal and are deposited at NHMD. Polymerase chain reactions were performed in 25  $\mu$ l reactions to amplify the mitochondrial cytochrome c oxidase I (COI) barcode region. The reaction consisted of 1  $\mu$ l of DNA extract, 4  $\mu$ l of 5 $\times$  HOT FIREPol Blend Master Mix Ready to Load With 10 mM  $MgCl_2$  (Solis BioDyne), 1  $\mu$ l each primer at 0.5  $\mu$ M and 18  $\mu$ l distilled water. The full barcode region of COI was targeted (658 bp, LCO1490 + HCO2198) using the following PCR cycle settings: 15' 94  $^{\circ}C$  – 5 $\times$  (30 s 94 $^{\circ}C$  – 40 s 47 $^{\circ}C$  – 1' 72 $^{\circ}C$ ) – 30 $\times$  (30 s 94 $^{\circ}C$  – 40 s 52 $^{\circ}C$  – 1' 72 $^{\circ}C$ ) – 7' 72 $^{\circ}C$ . Forward and reverse Sanger sequencing and purification of target genes was done by Macrogen (Amsterdam, Netherlands). The forward and reverse raw sequences were aligned to a reference *Lobrathium multipunctum* barcode (GenBank ID: KM447762) in Geneious Prime, edited for obvious alignment errors and the consensus was submitted to BOLDSystems. New sequences are available on BOLDSystems under accession numbers given in Table 1.

### Phylogenetic analyses

All generated COI sequences were aligned using the MAFFT Multiple Alignment v.1.4.0 plugin in Geneious (Kato et al. 2002). The alignment was partitioned by codon and submitted to PartitionFinder2 (Lanfear et al. 2017),

searching for best substitution models under the Bayesian Information Criterion with the following settings: models choice = all, branch lengths = unlinked and search choice = greedy. Two separate phylogenetic analyses were conducted using the best partition and substitution model found by PartitionFinder2: a Maximum Likelihood (ML) analysis using IQ-Tree v.1.6.10 (Nguyen et al. 2015) and a Bayesian analysis (BI) using MrBayes v.3.2.7a (Ronquist et al. 2012). The ML analysis in IQ-Tree was set up with default settings, except: Ultrafast Bootstrap (UFB) was run for 1000 iterations (-bb 1000), then re-run with up to 10,000 iterations (-nm 10,000) with a SH-aLRT test (-sh\_test true) and run for 1000 iterations (-alrt 1000). The BI analysis consisted of two runs of four chains each, with default settings, except that different rates of evolution were allowed in different partitions (ratepr = variable). Convergence was examined manually by checking the Potential Scale Reduction Factor (PSRF) in Tracer v.1.7.1 (Rambaut et al. 2018). For each analysis, we considered posterior probability values (PP)  $\geq 0.90$ , SH-aLRT  $\geq 80$  and UFB  $\geq 90$  to indicate clade support, respectively.

### Haplotype network and species delimitation analysis

An integer neighbour-joining (IntNJ) haplotype network was built using Popart 1.7.2 (Leigh and Bryant

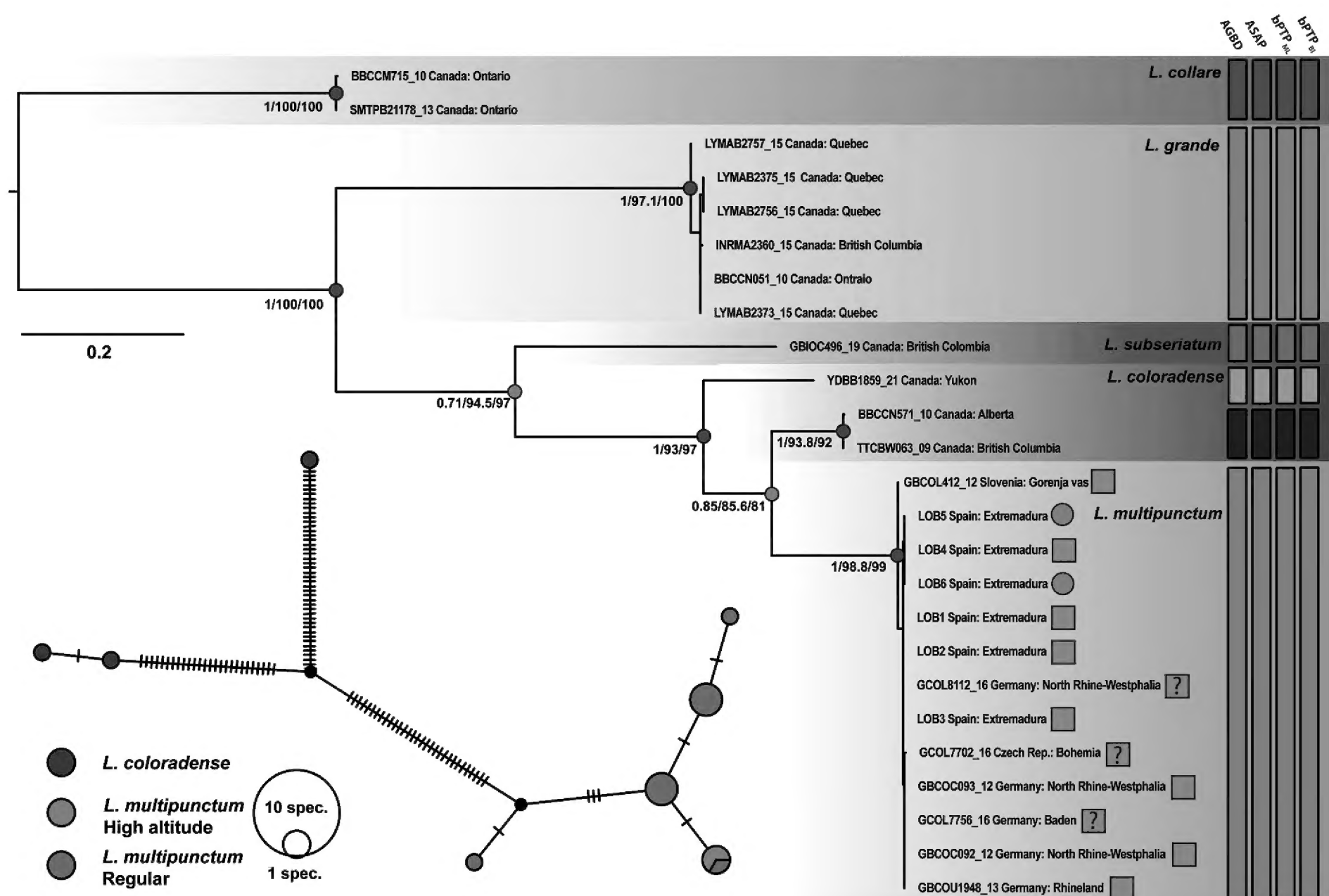


2015), based on aligned data from *L. coloradense* and *L. multipunctum*. Additionally, four separate species delimitation analysis were conducted. The first two analyses were performed using the FASTA alignment from our phylogenetic analysis (see above), the Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP), respectively (Puillandre et al. 2012; 2021). For the ABGD analysis, we used the default settings,  $P_{min} = 0.001$ ,  $P_{max} 0.1$ , Steps 10, and  $X = 1.5$ , through the ABGD web portal (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>). For the ASAP analysis, we used both the Jukes-Cantor (JC69) and Kimura (K80) model for substitutions. Additionally, a species delimitation analysis, based on tree inference, was performed on trees from our phylogenetic analysis (see above), the Bayesian Phylogenetic Species Concept (bPTP), based on our BI (bPTP<sub>BI</sub>) and ML (bPTP<sub>ML</sub>) tree, respectively (Zhang et al. 2013). Here an unrooted tree from each analysis (BI and ML) was imported using default settings, MCMC generations = 100000, thinning = 100, burn-in = 0.1. Output files were checked for convergence. The partitioning with the highest support for each method was mapped on to the phylogenetic tree as boxes with a gap when a cluster was discontinued.

## Results and discussion

During our field trip to Central Spain in and around the Sierra de Gredos and the Tagus Basin, we encountered two different morphotypes of *Lobrathium multipunctum*. The high altitude morphotype was found at the much higher elevations of the Sierra de Gredos (Fig. 1A), while the regular morphotype was found across several sites of the lower elevation river valley (Fig. 1B). The high altitude morphotype of *Lobrathium multipunctum* studied here was earlier considered a distinct species, *Lathrobium (Lobrathium) hispanicum* Doderö, 1916. The striking external differences initially led us to question their synonymy by Assing (2007).

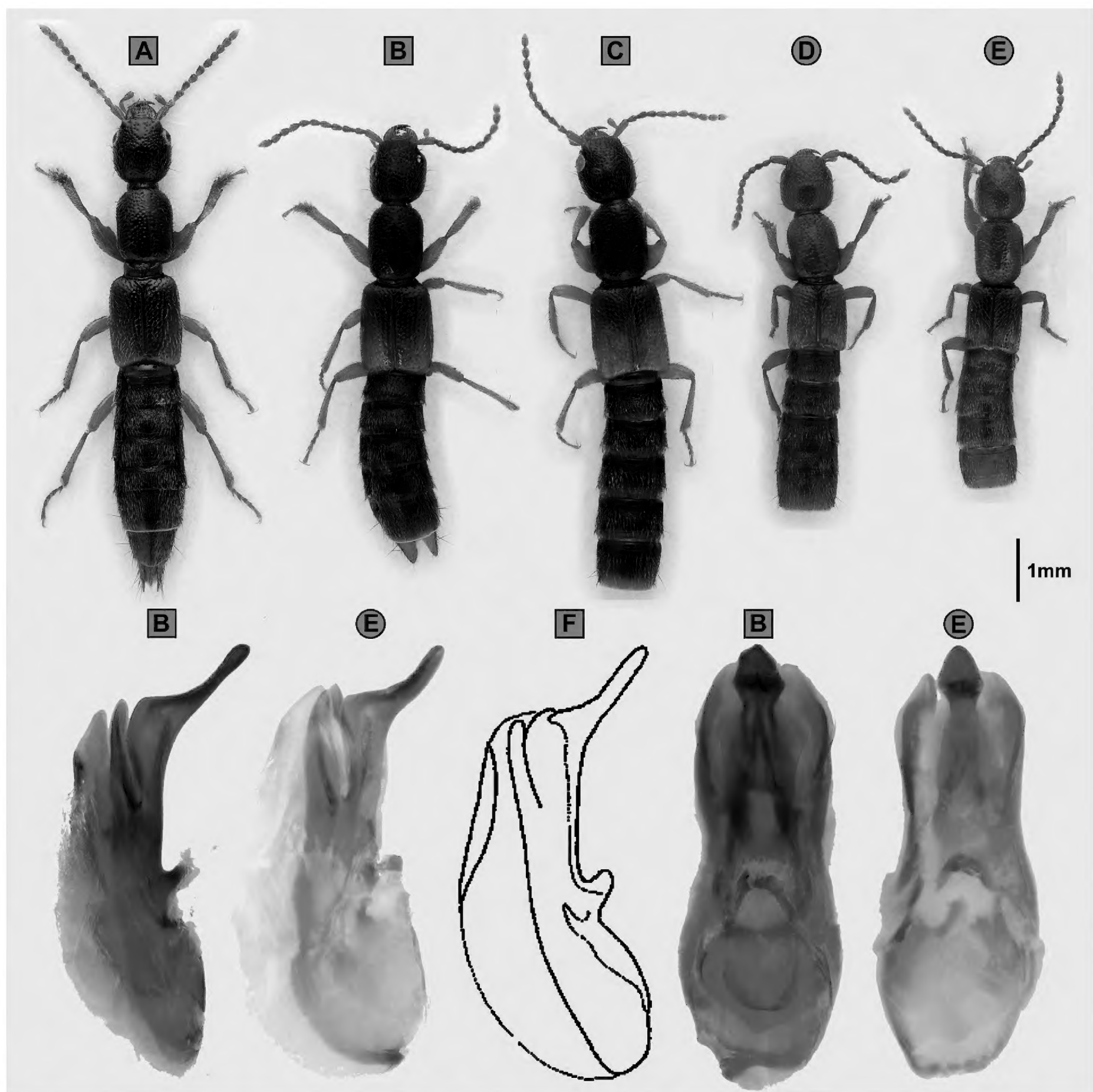
We were able to successfully sequence 658 bp COI barcodes of six individuals, two of the high altitude morphotype and four of the regular morphotype. These were analysed together with available data from GenBank and BOLD databases for *Lobrathium*. The final dataset included 25 COI barcodes belonging to five species, of which 13 were from the focal taxon. Both Maximum Likelihood and Bayesian Inference phylogenetic trees resulted in similar topology (Fig. 3). All species clusters, except for a single specimen of *Lobrathium coloradense* Casey, 1905, were recovered with high support and low intraspecific varia-



**Figure 3.** Consensus phylogenetic tree with support values from Bayesian and Maximum Likelihood analyses. Full support (green) is indicated by posterior probability values (PP)  $\geq 0.90$ , SH-aLRT  $\geq 90$  and ultrafast bootstrap (UFB)  $\geq 90$ , while partial support (yellow) is indicated by lower values. The most probable clustering of four different species delimitation methods is visualised by boxes with gaps between. An integer neighbour-joining haplotype network is presented bottom left. Size of circles show number of specimen in clusters and the line between the cluster represents a single nucleotide difference. High altitude morphotype (orange circle). Regular morphotype (purple square).

tion in both analyses, as well as all species delimitation analysis. This single barcode of *L. coloradense* was found away from the main cluster ( $n = 2$ ) of the species, which could be due to either a misidentification within the database or a potentially unexplored divergence to be studied in more detail. Based on the results of both ML and BI phylogenetic analysis and species delimitation analysis (Fig. 3), *L. multipunctum* was recovered monophyletic with strong support. We found the high altitude and regular morphotypes to be phylogenetically mixed, with almost no geographic variation (see haplotype map Fig. 3). Therefore, the results of our COI barcode phylogeny confirm the synonymy of Assing (2007), where *Lathrobium* (*Lobrathium*) *hispanicum* Doderö, 1916 was synonymised with *Lobrathium multipunctum* (Gravenhorst, 1802).

Assing (2007) also compared the morphology of these high altitude morphotypes to the regular ones and noted that, other than the apparent smaller size, pale colouration and shorter elytra brachypterous habitus, they also possessed a different structure of the punctation of the elytra (Fig. 4). The regular morphotype has the elytral punctures in clear rows, but in the high altitude morphotype, they are more scattered. Furthermore, he noticed that the genitalia are very comparable, which we were also able to observe and confirm (Fig. 4). There were slight differences, but these fall within variation of the species. We do not confirm the observation of Assing (2007) that the high altitude morphotype is brachypterous. All high altitude morphotypes studied by us had fully developed hind wings comparable to those of the regular morphotypes.



**Figure 4.** Habitus (top) and aedeagus (bottom) in lateral and parameral view of *Lobrathium multipunctum* (Gravenhorst, 1802). **A.** From Portugal; **B.** Voucher of LOB3; **C** Voucher of LOB1; **D.** Voucher of LOB5; **E.** Voucher of LOB6; **F.** Retraced from Assing and Schülke (2012). High altitude morphotype (orange circle). Regular morphotype (purple square).



It should be noted that the specimens studied here are not the only occurrence of high altitude morphotypes in *Lobrathium multipunctum*. Another two taxa, formerly regarded as distinct species (*Lobrathium endogeum* Coiffait, 1971 and *Lobrathium gallienii* Fagniez, 1917) were described from the Pyrenees and Central Massif, respectively and subsequently synonymised with *Lobrathium multipunctum*. *Lobrathium gallienii* was later re-instated by Assing (2019) based on the study of the male genitalia which was sufficiently different from *L. multipunctum*. Both taxa have similar external habitus to the specimens of the Sierra de Gredos, being small and pale in colour.

Our results highlight the presence of a high altitude morphotype amongst the widespread paederine rove beetle *Lobrathium multipunctum*. We hope our single example of a high altitude morphotype that is molecularly identical in the highly-used COI barcode to lowland conspecific amongst the mega diverse Staphylinidae (almost 70,000 species on the global scale (Newton 2022)) highlights the need for caution when describing high elevation endemics, especially based solely on COI barcodes. Ideally, where possible, taxonomic work should utilise DNA barcoding to confirm or refute the status of such taxa as full species or derived morphotypes adapted for particular altitudes, habitats or ecologies. Of course, such technological applications are not always possible, especially for the large body of citizen scientists who contribute a large amount of taxonomic knowledge in insects and other groups.

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